Human hemolysate contains several glycosylated minor hemoglobins (Hbs A\textsubscript{\textalpha\textalpha}, A\textsubscript{\textbeta\textbeta}, A\textsubscript{\textbeta\textgamma}, and A\textsubscript{\textgamma\textgamma}) which can be chromatographically separated from the major component, Hb A\textsubscript{\textcircled{0}}. The glycosyl-ketoamine linkage in Hb A\textsubscript{\textcircled{0}} can be detected colorimetrically by the thiobarbituric acid test. When chromatographed on Bio-Rex 70 resin, Hb A\textsubscript{\textcircled{0}} is eluted as a single peak following Hb A\textsubscript{\textalpha\textalpha}. We have found that the leading edge of Hb A\textsubscript{\textcircled{0}}, as well as Hb A\textsubscript{\textalpha\textalpha}, contains carbohydrate as detected by the thiobarbituric acid test. Both glycosylated components were comparably increased in the diabetic. There was a corresponding increase in the incorporation of tritium from [\textsuperscript{3}H]borohydride into diabetic Hb A\textsubscript{\textcircled{0}}. After Hb A\textsubscript{\textcircled{0}} was incubated with [\textsuperscript{3}H]glucose it was chromatographed on Bio-Rex 70 resin. The specific activity profile corresponded closely to the thiobarbituric acid test profile. Parallel incubation with glucose having \textsuperscript{3}H bound to the second carbon atom confirmed that both the synthetic Hb A\textsubscript{\textcircled{0}} and the glycosylated Hb A\textsubscript{\textcircled{0}} have undergone the Amadori rearrangement to the more stable ketoamine linkage. We estimate that 8 to 10\% of Hb A\textsubscript{\textcircled{0}} in normal red cells is glycosylated. Autoradiograms of tryptic peptide maps indicate that several sites on both the \alpha and \beta chains are modified, including the NH\textsubscript{2}-terminal amino groups which allows them to be effective nucleophiles at physiological pH. Accordingly, one might predict that this site on the \beta chain would also be glycosylated. Furthermore, the e-amino group of lysine residues, despite their relatively high pK\textsubscript{a} values, are also capable of forming aldimine and ketoamine adducts with glucose (9-11).

In this report, we demonstrate unexpected structural heterogeneity of the major human hemoglobin component (Hb A\textsubscript{\textcircled{0}}) which is due to the same kind of nonenzymatic glycosylation that characterizes Hb A\textsubscript{\textcircled{0}}.

**MATERIALS AND METHODS**

Blood specimens were obtained from normal volunteers and patients with diabetes mellitus. Red cell hemolysates, prepared by the method of Drabkin (12), were gassed with carbon monoxide and chromatographed on Bio-Rex 70 cation exchange resin (Bio-Rad, Inc., Calif.). Usually, 2 g of hemoglobin protein was applied to a 5 X 38 cm column. The non-hemoglobin protein and negatively charged minor components were eluted by a linear NaCl gradient going from 0.1 to 0.5 M. Column fractions containing isolated hemoglobin components were pooled and, when necessary, concentrated by pressure filtration (Amicon PM 10 membrane).

Poly(L-lysine) hydrobromide was purchased from Sigma Chemical Co. It had a molecular weight range of 30,000 to 70,000 and consisted of linear polymers of L-lysine linked by peptide bonds at \alpha-NH\textsubscript{2} and carboxyl groups.

**Incubations**—Purified Hb A\textsubscript{\textcircled{0}} (or in some cases polylysine) was incubated with uniformly labeled n-[\textsuperscript{3}C]glucose, n-[\textsuperscript{2}H]glucose, or with unlabeled p-glucose for up to 26 days in a sterile solution of Krebs-Ringer phosphate, pH 7.4, at 37°C. In one experiment, intact
Hemolysates were applied to a column (0.9 x 16 cm) of DC-GA resin by ion exchange chromatography as modified from Jones (18). The procedure described above and in Ref. 13.

Precipitation, and separated into \( \alpha \) and \( \beta \) subunits by carboxymethyl-

with a 200-fold excess of NaBH\(_4\) for 10 min at room temperature.

The tryptic peptides were analyzed by two-dimensional

maps on cellulose thin layer plates (16). Autoradiograms were prepared on X-omat film (Eastman Kodak Co.).

Reduced red cells were incubated with 12 mM L-[\(^{14}C\)]glucose in Krebs-Ringer phosphate \(^2\) and plasma (1:1) at 37°C for 7 days. Unbound glucose was separated from protein by passage through Sephadex G-25. The hemoglobin solutions were then analyzed by the chromatographic procedure described above and in Ref. 13.

Glucosyl valine (valyl-1-deoxyglucose) was prepared as described by Dixon (14) from \([^{14}C]\)valine and unlabeled glucose. The adduct was then reduced with unlabeled borohydride.

**Structural Analyses**—Glucose covalently bound to either hemoglobin or polysynine by ketoamine linkage was measured by treatment with oxalic acid to generate 5-hydroxymethylfurfural which was then condensed with thiobarbituric acid (TBA) to form a colored adduct, having an absorbance maximum at 443 nm (7). These results can be expressed as specific color activity (SCA\(^2\) or SCA-A/mg of protein) (see TBA test, Table I). Hemoglobin and glucosylated polysynine were treated with either unlabeled or \('H\)-labeled sodium borohydride. The enhanced stability of the amino-1-deoxysorbitol linkages enabled the carbohydrate to remain attached to the protein throughout the analytic procedures. In addition, the reaction with labeled borohydride was useful in the detection of aldimine and ketoamine linkages. The reductions were carried out as described by Bookchin and Gallop (5). Samples (0.3 to 1.0 mg (\(\alpha/\beta\) dimer) in 0.1 M potassium phosphate, pH 7.0) were reacted with a 200-fold excess of NaBH\(_4\), for 10 min at room temperature followed by 50 min at 10°C. Excess borohydride was removed by acidification to pH 5, followed by gel filtration on Sephadex G-25 at 4°C in either 0.01 M NH\(_4\)HCO\(_3\) pH 7.8 (polylysine) or 0.05 M Tris-acetate, pH 8.0 (hemoglobin). The polysynine samples were lyophilized.

Hemoglobin samples were converted to globin by acid/acetone precipitation, and separated into \( \alpha \) and \( \beta \) subunits by carboxymethyl cellulose chromatography in 8 M urea (15). Purified globin subunits were aminoethylated and digested with trypsin as described previously (16). The tryptic peptides were analyzed by two-dimensional maps on cellulose thin layer plates (16). Autoradiograms were prepared on X-omat film (Eastman Kodak Co.).

Globin subunits, glucosylated polysynine, and peptides were hydrolyzed in constant boiling HCl, 108°C, for 24 h. Some specimens were hydrolyzed in 2 N KOH and desalted by the method of Hauschka (17). Preparative separations of labeled amino acids were carried out by ion exchange chromatography as modified from Jones (18). Hydrolysates were applied to a column (0.9 x 16 cm) of DC-6A resin (Durrum Chemical Co., Sunnyvale, CA) and eluted with a 200-ml linear gradient of 0.2 M pyridine acetate, pH 3.1, to 1.5 M pyridine acetate, pH 5.0. Column pressure averaged 300 psi.

Peaks from this column were pooled and dried down at 60°C under nitrogen. They were then resolubilized in 1 ml of 0.4 M periodic acid in water to remove sugars so that the amino acid involved could be identified. After reacting for 2 h in the dark at room temperature, samples were lyophilized and subjected to amino acid analysis. Amino acid analyses were done on a Durrum D-500 analyzer by AAA Laboratories, Seattle, WA.

Aliquots of chromatographic fractions of hemoglobin, globin subunits, peptides, and amino acids were counted in a liquid scintillation counter (Isocap 300, Bealite Analytical) using Beckman Ready Solv liquid scintillation fluid. Counts were corrected using a standard quench curve.

**RESULTS**

**Glycosylation of Intact Hemoglobin**—The chromatograms from a normal and a diabetic hemolysate are shown in Fig. 1. As expected, the diabetic had about a 2-fold increase in Hb \( A_\text{b} \). This figure also shows the ketoamine-linked sugar detected by the TBA test (Ref. 7 and Table I). Hemoglobin-bound sugar was detected in the leading edge of the Hb \( A_\text{b} \) peak as well as in Hb \( A_\text{b} \).

**Glycosylation of Intact Human Hemoglobin**

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\(^1\) New England Nuclear Co., Boston, MA.

\(^2\) Krebs-Ringer phosphate contains 0.13 M NaCl, 0.013 M MgCl\(_2\), 0.008 M CaCl\(_2\), 0.05 M KCl, and 0.01 M sodium phosphate, pH 7.4.

\(^3\) The abbreviations used are: SCA, specific color activity; TBA, thiobarbituric acid.
ively (Table I). In addition, a lesser amount of color was developed in the leading edge of the major hemoglobin component. Hb Ao of the diabetic contained about twice as much total TBA color as the normal. These results suggest that the major hemoglobin peak contains ketoamine linked carbohydrate in proportion to the concentration of Hb Ao. A similar pattern was observed when the trailing edge of purified Hb Ao was incubated for 21 days with 15 mM $[^{14}C]$glucose (Fig. 2). In agreement with earlier studies (7, 13), Hb Ao was formed. This synthetic component contained 0.0 mol of glucose/$\beta$ chain. However, about 4 times as much $[^{14}C]$glucose was incorporated into the leading edge of Hb Ao as into Hb Aa.

The TBA test revealed considerably less specific color activity in the $[^{14}C]$glucose-Aa than in the $[^{14}C]$glucose-Ao (See Fig. 2 and Table I). Thus, the TBA test was several-fold less sensitive in detecting the glucosyl linkages in Hb Ao than in Hb Aa. This difference could not be explained by decreased hydrolysis.

**Table I**

Estimation of carbohydrate content of hemoglobins by the TBA test

<table>
<thead>
<tr>
<th>Hemoglobin</th>
<th>SCA</th>
<th>A/mg Hb</th>
<th>A/µmol αβ</th>
<th>A/µmol glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aa (normal)</td>
<td>0.130</td>
<td>4.18</td>
<td>4.42</td>
<td></td>
</tr>
<tr>
<td>Aa (diabetic)</td>
<td>0.169</td>
<td>5.40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aa (normal)</td>
<td>0.0019</td>
<td>0.061</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aa (diabetic)</td>
<td>0.0044</td>
<td>0.141</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Determined from unincubated (native) hemoglobins.
* Determined from hemoglobins following incubation of $[^{14}C]$glucose with purified Hb Ao.

![Fig. 3. Incubation of Hb Ao with D-glucose doubly labeled with $^{14}C$ and $^{3}H$ bound to the second carbon atom. The relative amounts of $^{3}H$ and $^{14}C$ bound to the labeled glucose solution are shown in the bar graph inset. After 22 days of incubation, $^{14}C$-labeled Hb Ao was formed as in Fig. 2. In addition, glucose was incorporated into the leading edge of Hb Ao (shown on a different scale). However, only a trivial amount of $^{3}H$ radioactivity was detected in either hemoglobin. The hydrogen atom bound to the second carbon atom was expelled when the glucose-hemoglobin adduct underwent the Amadori rearrangement from the aldime to ketoamine form.](http://www.jbc.org/)

Of the sugar from the hemoglobin during oxalid acid treatment prior to reaction with TBA. Eighty-one per cent of the radioactivity from the αβ was recovered after hydrolysis, compared with 97% for Aa. As the following experiment shows, the difference was also not due to failure of the glucose linkages in Aa to undergo the Amadori rearrangement.

In order to elucidate the nature of this linkage, we incubated purified Hb Ao with a mixture of [2-$^{3}H$]glucose and $[^{14}C]$glucose under the same conditions described above (see legend of Fig. 3 for details). The ratio of $^{3}H$ to $^{14}C$ radioactivity incorporated into hemoglobin provides an accurate measure of the Amadori rearrangement of the aldime to the more stable ketoamine linkage (7) as shown by the reaction scheme in the introduction. After 6 days of incubation, about 98% of the newly synthesized Hb Aa, and Hb Ao had undergone the Amadori rearrangement. As shown in Fig. 3, after 22 days, very little $^{3}H$ radioactivity was detected in either Hb Aa or Hb Ao. We estimate that 91% of the Hb Aa, and 93% of the glycosylated Hb Ao had undergone the Amadori rearrangement.

In order to determine whether glycosylation of Hb Ao and Hb Aa takes place in the intact red cell under controlled physiologic conditions, we incubated a sterile suspension of normal fresh human red cells with 12 mM L-$[^{14}C]$glucose for 2 days at 37°C. Considering that hemoglobin has a 2-fold axis of symmetry, the L isomer should interact with hemoglobin in the same manner as the $D$ isomer and yet would not participate in any enzymatic reactions such as glycolysis and the hexose monophosphate shunt. The radioactivity profile, shown in Fig. 4, indicates direct condensation of glucose with hemoglobin inside the intact red cell, resulting in the synthesis of Hb Aa. Incorporation of radioactivity into Hb Aa was also observed.

**Structural Analysis of Glycosylated Hb Ao—Hemoglobin Aa incubated with $[^{14}C]$glucose under physiologic conditions, incorporated radioactivity into Hb Aa and into the leading edge of Hb Ao (See Fig. 2). Following reduction with unlabeled borohydride, the labeled Hb Aa was separated into $\alpha$- and $\beta$-globin subunits. As the elution profile in Fig. 5 (top) shows, the radioactivity was evenly distributed between $\alpha$ and $\beta$ chains. The glycosylated species were eluted slightly ahead of the unreacted globin, indicating that they were slightly more negatively charged. When Hb Ao was reduced with $[^{3}H]$borohydride (without prior incubation with glucose), the elution...**

* We thank Dr. George Cahill for suggesting this experiment.
**Structural Heterogeneity of Human Hemoglobin**

**Fig. 4.** Incubation of intact red cells with L-[14C]glucose. Incubation conditions are described under "Materials and Methods." After 48 h, the red cells were lysed and the hemoglobin was chromatographed on Bio-Rex 70. The metabolically inert sugar was incorporated not only into Hb A1c, but also into Hb A0. Hemoglobin concentration, •—•, radioactivity, ○—○.

**Fig. 5.** Separation of globin α and β subunits on CM-cellulose in 8 M urea. **Top,** glycosylated Hb A0, following incubation with D-[14C]glucose and reduction with unlabeled borohydride. **Bottom,** Hb A0 following reduction with [3H]NaBH₄. Protein concentration is measured by A₂₈₀nm (•—•), while radioactivity is shown by ○—○. In each case, radioactivity was located in the leading edge of α and β chains.

The profile of the globin subunits showed a very similar pattern (Fig. 5, bottom) with maximal specific radioactivity at the leading edges of the α and β subunits. These results suggest that synthetic glycosylated Hb A0 has a distribution of ketamine-linked glucose similar to that of authentic Hb A0. Hb A0 from a diabetic individual had 2.6-fold more incorporation of tritium from [3H]NaBH₄, compared to normal Hb A0 (data not shown).

In order to determine which residues are glycosylated during incubation of Hb A0 with [14C]glucose, we treated the leading edge of labeled Hb A0 with unlabeled borohydride. The α and β subunits were isolated, acid-hydrolyzed, and subjected to amino acid analysis. As shown in Fig. 6, middle and bottom, a predominant radioactive peak was observed in a region following the elution of phenylalanine and preceding the elution of histidine, lysine, and arginine. The identification of this peak as a glucose-lysine adduct was established in two ways. First, synthetic lysino-1-deoxysorbitol was prepared by incubating polylysine with [14C]glucose under conditions similar to those employed in the hemoglobin incubations; after separation of the protein from free [14C]glucose by gel filtration, it was reduced with unlabeled borohydride and subjected to acid hydrolysis. The amino acid analysis in Fig. 6, top, shows that the synthetic lysino-1-deoxysorbitol eluted at precisely the same place as the 14C-labeled peaks obtained from the subunits of Hb A0. Secondly, periodate treatment of the radioactive peaks obtained from hydrolysates of polylysine and α-globin yielded unlabeled lysine. In contrast, the radioactive amino acid obtained following acid hydrolysis of synthetic Hb A1c eluted with the solvent front, as did synthetic valyl-1-deoxysorbitol. Following treatment of the glycosyl va-
line with periodate, 82% of the radioactivity was recovered as valine.

Hemoglobin $A_0$ was isolated from normal (unincubated) hemolysate on Bio-Rex 70 (13) and then treated with $[^3H]$borohydride without any prior incubation. Following acid hydrolysis of the separated $\alpha$ and $\beta$ chains, the amino acids were separated as above. As shown in Fig. 7, a prominent $^3$H-labeled peak corresponding to lysino-1-deoxysorbitol was found following acid hydrolysis of the isolated $\alpha$ chain. In contrast, the elution pattern of the $\beta$ chain consistently revealed considerable heterogeneity with several radioactive peaks besides lysino-1-deoxysorbitol (Fig. 7). Similar heterogeneity was seen following alkaline hydrolysis of the $\beta$ chain. Apparently, the $\beta$ chain takes up tritium at sites other than glucose-lysine adducts. This nonspecific uptake of tritium was circumvented when Hb $A_0$ from a diabetic was reduced with $[^3H]$borohydride. As Fig. 8 shows, the acid hydrolysate from normal $A_0$ globin contained several labeled peaks besides that corresponding to lysino-1-deoxysorbitol while a comparable preparation of $A_0$ globin from a diabetic showed a predominant lysino-1-deoxysorbitol peak. Diabetic Hb $A_0$ contains relatively more covalently bound glucose compared to normal Hb $A_0$. These results demonstrate the presence of the glucose-lysine adduct not only in Hb $A_0$ incubated with glucose but also in naturally occurring Hb $A_0$.

Following incubation of Hb $A_0$ with $[^14C]$glucose and reduction with unlabeled borohydride, the $\alpha$- and $\beta$-globin subunits were isolated, digested with trypsin, and then analyzed by two-dimensional peptide mapping on cellulose thin layers. Fig. 9 shows the ninhydrin-stained fingerprints of the $\alpha$ and $\beta$ chains and the corresponding autoradiograms. The $\beta$ chain contained several prominent radioactive peptides, none of which correspond precisely with ninhydrin-positive peptides. Since only a small proportion of the $\beta$ chains was glycosylated at each of these sites, it is not surprising that they were not detected by ninhydrin. The $\alpha$ chain, shown in Fig. 9A, contained one prominently labeled peptide. Analysis of this peptide following purification indicated that it was $\alpha$-Tp-1 with a blocking group at the NH$_2$-terminal valine. In addition, several other radioactive $\alpha$ chain peptides were also noted. The relative intensity of $^{14}$C-labeled $\alpha$-Tp-1 varied considerably with the sampling of hemoglobin from the leading edge of the Hb $A_0$ peak. The hemoglobin that was eluted initially (and comprised the shoulder of TBA-positive material in Fig. 2) was relatively rich in glycosylated $\alpha$-Tp-1 (Fig. 7B) while the relative amount of this labeled peptide decreased considerably when a larger portion of the labeled Hb $A_0$ was analyzed. In view of the amino acid analyses shown in Figs. 6 and 7, the remaining labeled peptides of the $\alpha$ chain and all those of the $\beta$ chain are likely to be glycosylated at the e-amino group of lysine residues. It is unlikely that trypsin cleaves at these lysino-1-deoxysorbitol residues (19). We are currently attempting to prepare sufficient amounts of these purified labeled peptides for structural analysis.

**Relative Stabilities of Hb $A_0$ and Glycosylated Hb $A_0$**—Hb $A_0$ was incubated with 15 mM $[^14C]$glucose for 15 days as described under "Materials and Methods." Following dialysis, the radioactive Hb $A_0$ and Hb $A_0$ peaks were isolated and reincubated under identical conditions in the presence of 15 mM unlabeled glucose for 5 additional days. No radioactivity was lost from Hb $A_0$ during this period. The content of

![Fig. 7. Separation of labeled amino acids as in Fig. 6. Normal Hb $A_0$ was reduced with $[^3H]$borohydride, separated into $\alpha$- and $\beta$-globin subunits, and then hydrolyzed. Top, $\alpha$ chains; bottom, $\beta$ chains.](http://www.jbc.org/)

![Fig. 8. Separation of labeled amino acids as in Fig. 6. Top, polylysine (PL) after incubation with unlabeled glucose followed by reduction with $[^3H]$borohydride and acid hydrolysis. Middle, normal globin reduced with $[^3H]$borohydride and acid hydrolysis. Bottom, diabetic globin treated in the same manner.](http://www.jbc.org/)
Structural Heterogeneity of Human Hemoglobin

Estimation of the Extent of Glycosylation of Hb Ao—The moles of glucose bound per αβ dimer of normal unincubated Hb Ao were estimated by two independent approaches: *in vitro* biosynthesis and the TBA test. 1) When Hb Ao was incubated with [14C]glucose sufficient to generate the percentage of Hb Ao found in normal hemolysate, 12 to 14% of Hb Ao became glycosylated. 2) When analyzed by the TBA test, glucose covalently bound to Hb Ao generates much less color compared to Hb Ai, (Fig. 1 and Table I). The TBA SCA (A/mg of protein) of Hb Ao is about 1.5% that of Hb Ai,. By performing the TBA test on Hb Ao, which has been incubated with [14C]glucose, we found an average SCA of 0.727 (A/μmol of glucose) compared with 4.42 (A/μmol of glucose) in the Ai, formed during the same incubation. Thus, the efficiency of color development for the glucosyl-lysine linkages in Hb Ao appears to be 6.1-fold lower than for the glucosyl-valine linkage in Hb Ai,. The SCA for Hb Ai, formed during this incubation was very close to that of native Hb Ai,. Thus, the TBA profile in Fig. 1 gives a falsely low impression of the amount of glucose covalently bound to Hb Ao. Correcting the SCA of Ao in normal hemolysate by the same factor, we arrive at an estimation of 9% for the amount of glycosylation in Ao. In light of our evidence that the glucose linkages in Ao involve lysines, the differential SCA (A/μmol of glucose) between Ao and Ai, can be further examined by conducting the TBA test on polylsyne incubated with [14C]glucose. In this case, we find a SCA of 0.603 (A/μmol of glucose) on polylsyne, about the same as that observed for the incubated Ao. We conclude that normal Hb Ao has an average of 0.09 to 0.13 mol of glucose/mol of αβ dimer. Most of the sugar is bound to the ε-amino groups of several lysine residues on both α and β chains. The fact that lysino-1-deoxyfructose linkages in Hb Ao appear to be slightly less stable than the valyl-1-deoxyfructose linkage in Hb Ai, tends to favor the lower estimate.

In their structural analysis of Hb Ao, Bookchin and Gallop (5) found that following reduction of the purified minor component with [3H]NaBH₄, the α chain contained about 10% of the radioactivity of the β chain. We have obtained similar results which can be explained by the presence of these additional glucose-lysine adducts.

DISCUSSION

It has generally been assumed that chromatographically pure human hemoglobin A (Hb Ao) is homogeneous. Our studies reveal unexpected structural heterogeneity in Hb Ao due to the linkage of glucose to the ε-amino group of lysine residues on both α and β subunits as well as to the NH₂-terminal valine of the α chain. The lysino-1-deoxyglucose linkages undergo the Amadori rearrangement to the ketoamine, lysino-1-deoxyfructose, identical to the structural transition at the β-NH₂ terminal in Hb Ai, (Fig. 3 and Ref. 6). Furthermore, the adducts formed at these lysine residues are nearly as stable as that at the β-NH₂ terminus. The comparison of normal versus diabetic red cells (Fig. 1), as well as the *in vitro* incubations with [14C]glucose (Fig. 2), show that the glycosylation of Hb Ao increases in parallel with the formation of Hb Ai,. Gabbay et al. (20) have compared a large number of normal and diabetic individuals and have found that the amount of sugar attached to Hb Ao, as detected by the TBA test, correlates with the level of Hb Ai,. These results indicate that glycosylation of Hb Ao is also a slow, nonenzymatic process reflecting the average intracellular blood glucose level.

Normal human hemolysate contains about 4% Hb Ao. In addition, we estimate that Hb Ao contains about 0.1 glucose...
molecules per αβ dimer. In determining this relationship, two independent approaches were used, both of which are indirect and subject to considerable analytical error. From these estimates, we conclude that in normal red cells, the β-NH₂ terminus is the site most frequently glycosylated (0.04 mol/αβ). The α-NH₂ terminus is a somewhat less common site of attack. In contrast, the much lower probability of glycosylation of individual lysine residues is probably due to the relatively high pKₐ of the ε-amino group.

Even though glucose binds covalently to a number of different amino groups on the α and β chains, only one modification, at the β chain NH₂ terminus, results in the formation of a chromatographically distinct minor hemoglobin component (Hb A₀). Glycosylation of amino groups, when followed by rearrangement to the ketoamine form, does not remove the charge from these groups, but does lower their pKₐ values. Chromatography at neutral pH, near the pKₐ values of the α-amino groups, optimizes the chance of resolving hemoglobin glycosylated at the NH₂ terminus of the β chain and probably explains the separation of Hb A₀ as a discrete peak. By use of a shallow salt gradient, we have been able to enrich hemoglobin glycosylated at the α chain NH₂ terminus to the leading edge of the Hb A₀ peak, but have been unable to isolate it as a chromatographically distinct minor component. Thus, the change in pKₐ at this site due to glycosylation is slight. In the case of hemoglobin glycosylated at lysines, even a moderate change in pKₐ would not result in a significant separation of this component from the main Hb A₀ peak since the chromatography is performed 4 pH units below the normal pKₐ of the ε-amino groups. Thus, Hb A₀ differs from the other glycosylated components in two respects: it is the most abundant and the glycosylation at this particular site happens to confer a decrease in isoelectric point in a pH range which enables it to be separated from the major component by chromatographic and electrophoretic methods.

These results argue strongly against specificity in the interaction of glucose with hemoglobin. In contrast, glucose 6-phosphate attaches specifically to the NH₂ terminus of the β chain because its phosphate group serves as an affinity label, leading the molecule to the diphosphoglycerate binding site (21). The relative ubiquity of the glucose-hemoglobin interaction suggests that other proteins are modified in a similar fashion. It has long been recognized that α- and ε-amino groups on protein can form adducts with glucose during in vitro incubations (22-24). Good candidates for this type of nonenzymatic post-translational modification in vitro include proteins which have relatively long turnover times and are exposed to high glucose concentrations found in plasma. Tanzer et al. (9) have demonstrated hexose attached to the ε-amino of hydroxylysine in bovine collagen while Bailey and his colleagues have found hexose-ε-amino lysine both in collagen (25) and in proteins of the normal erythrocyte membrane (10). Recently, preliminary results have suggested the presence of glucosyllysine in basic myelin protein of nerve (26) and also in crystallin following the incubation of lens with glucose (11). There is considerable interest in the possibility that this post-translational modification may contribute to the pathogenesis of the long term complications of diabetes.

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